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Use of *Mycobacterium avium* subsp. *paratuberculosis* specific coding sequences for serodiagnosis of bovine paratuberculosis

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ABSTRACT

In this study, the finished complete genome of *Mycobacterium avium* subsp. *paratuberculosis* (Map) was screened for specific coding sequences that could be very valuable in the design of a sensitive and specific Map detection serological assay. Eighty-seven Map-specific sequences were retained. Among these, three candidate antigens have been analysed for their serodiagnostic potential. These antigens were selected on the basis of their putative immunogenicity as predicted by *in silico* analysis. The antigens were cloned in *Escherichia coli*, expressed, and purified before testing in an antibody detection ELISA test, using a well characterized panel of 18 and 48 sera from Map infected and uninfected cattle, respectively. Two of these antigens, antigen 6 and MAP1637c, yielded in our conditions a sensitivity of 72% and 82%, respectively, for a specificity of 98%. It is particularly noticeable that, when probed with the same serum panel, the most widely used European paratuberculosis commercial seroassay (Pourquier test) yielded a sensitivity of 72% for a specificity of only 92%.

1. Introduction

Bovine paratuberculosis is a chronic granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map) and characterised by intermittent diarrhoea associated with rapid weight loss. Variable prevalence has been reported (Cetinkaya et al., 1996; Giese and Ahrens, 2000; Jakobsen et al., 2000; Kennedy and Benedictus, 2001; Streeter et al., 1995; Wells and Wagner, 2000), ranging between 3.5% and 70% in developed countries, and annual financial losses (Gonda et al., 2007) were evaluated to $200 million for the US diary industry alone (Ott et al., 1999).

It is largely accepted that major improvements are needed in currently available serodiagnostic tests to allow for an efficient control program to be set up (Collins et al., 2005; Sweeney et al., 2006). Many laboratories have therefore chosen to improve the efficiency of serological assays by replacing the current common "extract antigen" by "purified Map-specific subunit antigens" (Dupont et al., 2005; Huntley et al., 2005; Olsen et al., 2001; Paustian et al., 2004; Willemsen et al., 2006). Different approaches have been used in the search for such antigens. Among other things we can mention immunoproteomic approaches, in which antigens are firstly selected on the basis of their antigenicity, and genomic approaches, in which antigens are selected in the finished genome of Map on the basis of their sequence specificity (Li et al., 2005). Recently, two large-scale immunoproteomic analyses have been published and reported very promising results

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obtained via such approach (Cho et al., 2006, 2007; Leroy et al., 2007). To date, a number of antigens have also been identified using the genomic approach, but data about their reactivity in ELISA using large panels of sera, are still lacking (Bannantine et al., 2004a, 2004b, 2008a).

Here, we present data on the bioinformatic screening of the Map genome in search for Map-specific genes. Among them, Map-specific coding sequences predicted to present the most potent antigenicity, as determined *in silico*, were selected for cloning, expression and serological analysis. Three candidate antigens were selected and probed in an ELISA test with 66 sera from Map-infected cattle and negative control cattle. Two of these candidate antigens were strongly antigenic and might be included in a diagnostic assay based on purified antigens.

2. Materials and methods

2.1. Sera

Sera used in this study have already been extensively described previously (Leroy et al., 2007). Briefly, a panel of 48 sera from two *M. bovis* infected herds with no known cases of paratuberculosis were used as negative controls. All animals included as negative controls were tested in the *in vitro* interferon gamma assay using ESAT6 and CFP10 and post-mortem by bacterial culture. Most had their *M. bovis*-sensitised status confirmed by the interferon gamma test (41/48), or by post-mortem *M. bovis* isolation (20/48). 18 sera originating from naturally Map infected cattle were used to study antigens sensitivity. These animals were 3–5 years old and were either low (5), medium (7) or high (6) faecal shedders of Map at time of sampling. Three high faecal shedders showed clinical signs of JD and 2 out of 4 sacrificed high shedders demonstrated extensive intestinal lesions. For all 18 positive controls, Map infection was confirmed by faecal culture. Both positive and negative serum panels were tested with the Pourquier test as reference serological assay. All data about the sera and results of Pourquier test are summarised in supplemental Table SI.

2.2. Selection of candidate antigens

Before the availability of the genome of Map [completed since February 2004 (*Mycobacterium avium* subsp. *paratuberculosis* str. k10/Li, L., Bannantine, J., Zhang, Q., Amonsin, A., Alt, D. and Kapur, V. Direct submission. Submitted on 5 September 2003 and modified on 30 January 2004)] it was already feasible to blast against the available Map contigs on the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi). The genome of *Mycobacterium avium* subsp. *avium* (Maa), that is the closest genome to Map, was used as query sequence for a BLASTn against the Map contigs. As a result all the Map contigs were returned and assembled in a unique silico-genome of 4,950,895 bases. In this case, borders of all joined contigs were replaced by N.

Coding sequences (CDS) were detected by the program GLIMMER. This program tracks down the stop codon of all genes and predicts a corresponding CDS. GLIMMER was used because it was not stopped by the unknown (N) bases. The smallest sequence recognised by GLIMMER is 90 bp long. Other programs were used to predict the structure and the localisation of the putative proteins. We used PsortII for Gram positives, TMHMM and TMPred programs.

A last selection was carried out using three different T cell epitope prediction programs, "Tsites", "BIMAS" and "SYFPEITHI", in order to select the most promising antigenic sequences, with respect to future vaccine development.

2.3. Production of the candidate antigens

Genes encoding candidate antigens were amplified by PCR from *M. avium* subsp. *paratuberculosis* ATCC 19698 genomic DNA (Rosseels et al., 2006) and initially cloned in a V1J.ns-tPA vector (Merck Research Laboratories, PA, USA) (Roupie et al., 2008). Coding sequences were subsequently subcloned by PCR amplification, (Expand High Fidelity PCR System, Roche) and purified on agarose gel (QIA quick Gel extraction kit, Qiagen). The amplified sequences were digested with BglII/HindIII (Ag5), with BamHI/HindIII (Ag6), and with BamHI/PstI (Ag7), purified on column (QIAkit PCR kit, Qiagen), and ligated into a pQE-80L (Qiagen) expression vector predigested with BamHI/HindIII (Ag5 and Ag6) or with BamHI/PstI (Ag7). After ligation (T4 DNA ligase, Fermentas) and transformation into Top-10F' chemically competent *Escherichia coli* cells (Invitrogen) for expression, positive clones were screened on LB-ampicillin medium (100 μg/ml) and confirmed by restriction enzyme digestion. The integrity of cloned sequences was checked by sequence analysis.

For antigen expression, *E. coli* positive clones were grown on LB-ampicillin medium at 37 °C under agitation until they reached an $OD_{600\ nm}$ of 0.6 and antigen expression was induced by addition of IPTG (1 mM final concentration) for 4 h for Ag6, and overnight for Ag5 and Ag7.

Bacteria were lysed using lysozyme (r-lysozyme, Novagen) and bath sonicator (2×5 min on ice) in extraction buffer (EB: 50 mM Tris–HCl, pH 7.5, NaCl 500 mM, Imidazole 10 mM, β-MSH 1 mM) containing 8 M urea and protease inhibitors (EDTA free miniprotease inhibitor tabs, Roche). Extraction efficiency of the different supernatants was analysed by SDS-PAGE.

2.3.1. Purification of candidate antigens

His-Select cartridges (Sigma) were used for candidate antigen purification following manufacturer's instructions. Candidate antigens were eluted using an imidazole gradient of 10–300 mM in 100 ml. To evaluate their homogeneity, concentrated candidate antigens were analysed in SDS-PAGE and all Coomassie Brilliant Blue stained bands were analysed by mass spectrometry. Protein concentrations were measured using the Non-Interfering Protein Assay kit (Novagen) following manufacturer's instructions.

2.4. Protein characterisation by mass spectrometry analysis

The Coomassie stained proteins of interest were cut out, and destained and washed twice in 25 mM NH_4HCO_3 for 15 min under gentle agitation at room temperature, followed by two 15-min washes in 25 mM NH_4HCO_3, 50% (v/v) CH_3CN. Cysteines were carboxyamidomethylated by

incubation of gel pieces in 25 mM NH_4HCO_3, 50 mM DTE for 30 min at 56 °C followed by 30 min in 25 mM NH_4HCO_3, 50 mM iodoacetamide at room temperature in the dark. After speed vacuum dehydration, 10 μl of a 0.02 μg/μl trypsin solution in 25 mM NH_4HCO_3 (Promega, Madison) was added, and samples were incubated overnight at 37 °C. Tryptic digestion was stopped by addition of 1 μl of 5% (v/v) formic acid. For MALDI-TOF analysis, 1 μl of each sample was mixed with 1 μl of matrix (5 mg/ml α-cyano-4-hydroxycinnamic acid and 0.5 pmol/μl rennin as internal standard, in 25% (v/v) ethanol, 25% (v/v) acetonitrile, 0.05% (v/v) TFA), then spotted onto a MALDI sample plate and allowed to air dry. MALDI-TOF was performed using a M@ldi™ mass spectrometer (Micromass, Manchester, UK) equipped with a 337 nm nitrogen laser. The instrument was operated in the positive reflectron mode with 15 kV of source voltage, 2.5 kV of pulse voltage and 2 kV of reflecting voltage. Protein identification was realised using ProteinLynx global server (Micromass) in an in-house Map genomic database, using mass tolerance of 100 ppm, carbamidomethylation of Cysteine as fixed modification and oxidation of methionine as variable modification.

2.5. Antibody ELISA

The commercial Pourquier ELISA was performed following the manufacturer's instructions. Candidate antigens were tested in ELISA on 96 well plates (MaxiSorb, Nunc) coated overnight at room temperature with 100 μl of candidate antigens diluted at 4 μg/ml in 100 mM Na_2CO_3/$NaHCO_3$ buffer, pH 9.6, or in formaldehyde 37% (v/v). After washing three times in PBS containing 0.05% (v/v) Tween 20 (PBST), non-specific interaction sites were blocked by incubation with 150 μl of 2.5% caseine hydrolysate (w/v) in PBST. 100 μl of sera diluted in PBST containing 1.25% caseine hydrolysate (w/v) (1:250) were then added and incubated 1 h at 37 °C. After washing three times with PBST, plates were incubated 1 h at 37 °C with 100 μl of secondary antibody diluted in PBST containing 1.25% caseine hydrolysate (w/v). Two different HRP conjugated secondary antibodies were used. The first one purchased from Sigma–Aldrich and raised against bovine Ig was diluted 1:2000. The second one is a monoclonal antibody specifically directed against type G bovine immunoglobulins. This antibody developed by Letesson et al. (1985) was kindly provided by Bio-X Diagnostic (Jemelle, Belgium) and diluted 1:50. After washing three times with PBST, HRP activity immobilised on the plate was detected by reaction with tetramethylbenzidine for 5–15 min. Reaction was stopped by addition of an equal volume of 1N sulphuric acid and evaluated by colorimetric measurement at 450 nm. ROC analysis was used to determine best cut-off value and associated sensitivity and specificity.

3. Results

3.1. Selection of candidate antigens

7137 CDS were predicted by GLIMMER and submitted to a BLAST 2.2.3. n (with an e value $<10e^{-3}$) against the genome of *Mycobacterium avium* subsp. *avium* (Maa).

After the BLAST, 285 CDS appeared absent from Maa. Ten sequences were considered as wrong because they overlapped two contigs. We translated the remaining 275 CDS into protein sequences and used a BLAST 2.2.3. p/nr.: 101 sequences with a hit on proteins from other mycobacteria were discarded. The ensuing selection of candidate antigens was based on the analysis of the 174 remaining sequences: first, we discarded two sequences that were overlapping each other, and second the sequences that were smaller than 120 bp were also discarded. Finally we got 121 sequences: 27 with "No significant similarity found", and 94 with one or more hits but without a BLAST mycobacterial hit.

Nineteen sequences were discarded because already analysed in other publications (Bannantine et al., 2002, 2004b).

To continue this selection, the programs Psort II and TMHMM were used to find the subcellular localisation of the protein. Proteins predicted to have more than three transmembrane helices were also discarded.

Finally, 87 putative proteins specific for Map were identified by this *in silico* method (Table SII): 25 sequences belonged to the annotated proteins of Map; 22 sequences had a BLAST with a result: "No significant similarity found"; and 40 putative proteins had a positive BLASTp but without a hit for other mycobacteria.

The antigenicity-driven selection based on three online available T cell epitope prediction programs (Tsites, BIMAS and SYFPEITHI) permitted finally to select three sequences as the putatively most potent to be analysed for antigenicity in an antibody ELISA test (Table 1). Sequence of Antigen 7 (coordinates: 1791069–1792508) corresponds to MAP1637c. Sequence of antigen 6 (coordinates: complement 886138–886530) has not been annotated in itself but it overlaps two sequences annotated on the complementary strand, MAP0864 (coordinates: 886023–886448) and MAP0865 (coordinates: 886451–887722). Sequence of antigen 5 (2383504–2384184) has not been annotated.

3.2. Production of candidate antigens

The three antigens were successfully cloned into the pQE-80L expression vector (QIAGEN), and expressed in *E. coli* as histidine-tagged proteins (data not shown). For an unknown reason, overnight expression was not suitable for Ag6 and was replaced by a 4 h induction It is possible that Ag6 presents some toxicity that would impair its overnight expression. All three antigens were expressed as inclusion bodies and required urea extraction. Moreover, Ag5 was only partially extracted in urea and use of detergent was necessary to obtain satisfactory yields.

3.3. Purification of candidate antigens

First, purified candidate antigens were analysed by mass spectrometry to confirm that the produced proteins matched the selected specific nucleotide sequences. For antigens 5 and 6, peptide mass fingerprints of tryptic digests corresponded to the right sequences. For antigen 7, on the other hand, in addition to a faster than expected

Table 1
Coordinates of the three selected sequences on Map chromosome.

	NCBI reference	Protein name	Location	pCDS on NC_002944.2
Antigen 7	NP_960571.1	MAP1637c	Complement	1791402–1792508
Antigen 6	N.D.	N.D.	Complement	886138–886530
Antigen 5	N.D.	N.D.	Complement	2383504–2384184

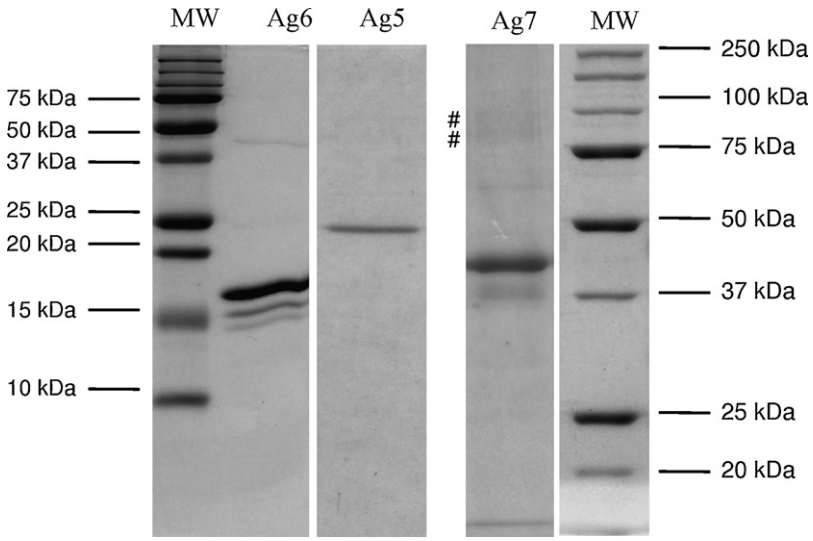

Fig. 1. SDS-PAGE analysis of purified candidate antigens. Purification efficiency was very high thanks to the use of an imidazole gradient for elution instead of the generally used step procedure. Contamination was monitored by mass spectrometry of all visible bands in the sample. Except for two unidentified bands in Ag7 (#), all other protein bands belonged to the studied proteins and resulted from degradation/polymerisation phenomena.

migration in SDS-PAGE, no peptides were identified in the N-terminal region of the purified protein. Sequencing of the cloned insert reveals the loss of this particular region. Only corrected coordinates are presented in Table 1.

IMAC strategy using an imidazole gradient rather than the common step procedure was particularly efficient for antigen purification. Indeed, purified and concentrated antigens were analysed by SDS-PAGE and all Coomassie stained bands were analysed by mass spectrometry (Fig. 1). In this analysis, all proteins bands were identified as belonging to the candidate antigens studied and resulting from polymerisation/degradation phenomena. Two weak protein bands in Ag7 sample (indicated by # in Fig. 1) were not derived from this antigen and were no *E. coli* contaminants either. Purification yields were in the range of 0.3–0.6 mg of candidate antigens/g of bacteria.

3.4. Serological assay

The three candidate antigens were tested individually for their ability to discriminate Map infected animals from the negative controls originating from two *M. bovis* infected herds. Most of the negative controls had their *M. bovis*-sensitised status confirmed by the ESAT6- and CFP10-based interferon gamma test or by post-mortem *M. bovis* isolation. Two different coating conditions were tested as well as two different secondary antibodies. Whatever the plate coating conditions, Ag5 gave the worst results. Indeed the discrimination that could be made between the responses obtained with sera from Map infected animals and from the negative controls was very poor [Se: 27.8% (9.70–53.48%, 95% CI); Sp: 97.92% (88.93–99.95%, 95% CI)]. Clearly, this antigen has no potential as diagnostic antigen since it failed in discriminating Map infected cattle (Fig. 2).

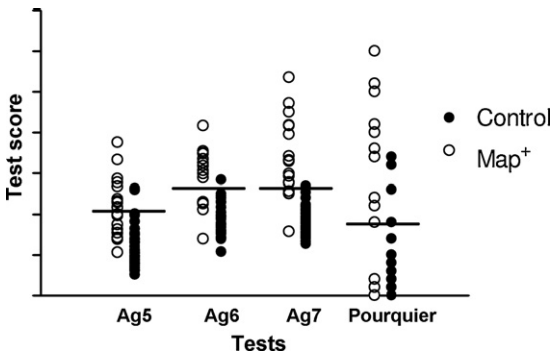

Fig. 2. Comparison of results obtained using Pourquier and Ag5-, Ag6- and Ag7-based ELISA. For Ag5, no significant difference was found between signals obtained with control, and MAP$^+$ sera (Se: 27.8%; Sp: 97.92%). On the other hand, sensitivities of 72.2% (46.52–90.31%, 95% CI) and 77.8% (52.36–93.59%, 95% CI) were obtained in four independent experiments realised with Ag6 and Ag7, respectively, with a constant specificity of 97.9% for both. These results clearly suggest that Ag6 and Ag7 could be valuable tools for development of an ELISA-based diagnostic test of Map infection in cattle.

Results obtained with Ag6 and Ag7 using coating in formaldehyde were only slightly better, resulting in sensitivities of 44.4% (21.5–67.4%, 95% CI) and 66.7% (44.9–88.4%, 95% CI), respectively, at a given specificity of 97.9% (93.9–100%, 95% CI) (data not shown). However, in contrast to what has been observed in previous studies (Leroy et al., 2007; Speer et al., 2006), sensitivity was increased by using a carbonate buffer for plate coating. Indeed, as shown in Fig. 2, sensitivities of 72.2% (46.52–90.31%, 95% CI) and 77.8% (52.36–93.59%, 95% CI) were obtained in four independent experiments carried out with Ag6 and Ag7, respectively, at a constant specificity of 97.9% for both (Fig. 2). For Ag7, the sensitivity could be further increased by use of an IgG-specific monoclonal antibody from Bio-X Diagnostic. Indeed, use of this secondary antibody permitted to enhance the sensitivity of the test to 83.3% for a specificity of 97.9% (data not shown). For Ag6, responses were not systematically increased using this Mab.

All these results are particularly interesting when compared to results obtained with the Pourquier test in a duplicate analysis (Table SI). Pourquier test when used to probe our panel of sera yielded a sensitivity of 72.2% (46.52–90.31%, 95% CI) for a specificity of 91.7 (83.8–99.5%, 95% CI) (Fig. 2). It thus seems that Ag6 and Ag7 performed better separately than the Pourquier test in discriminating Map infected cattle from negative controls originating from *M. bovis* infected herds. Nevertheless, no statistical difference between both tests was observed using a McNemar's test (data not shown).

4. Discussion

Mycobacterium avium subsp. *paratuberculosis* (Map) is the etiologic agent of bovine paratuberculosis, also called Johne's disease. As no effective therapeutics presently exist to combat Johne's disease, and because current vaccines interfere with control programs for bovine tuberculosis, large-scale testing and confinement of infected animal is the only reasonable way to limit disease prevalence in cattle (Harris and Barletta, 2001). However, despite several years of development, currently available diagnostic tests still lack performance to be used in large-scale control programs. Among the different available diagnostic tools (PCR, faecal culture etc.), serodiagnosis is the most promising. Indeed, ELISA-based serological tests are easy to perform, cheap to produce and do not require dedicated facilities. In these current commercial ELISAs, antigens used for plate coating are different, crude bacterial extracts requiring *M. phlei* pre-absorption of sera in order to remove cross-reactive antibodies. This pre-absorption step is not only time consuming but also affects test sensitivity (McKenna et al., 2005; Sockett et al., 1992). This is the main reason why we have chosen to focus our effort on the development of a serodiagnostic test based on one or a handful of purified antigens rather than on a Map extract.

In this study, a genomic analysis of Map genome allowed us to identify *in silico* Map-specific candidate antigens. A similar approach has already been used by other authors using approximately half of the *M. avium* subsp. *paratuberculosis* genome (Bannantine et al., 2002, 2004b). On the contrary we used almost all the Map genome to perform our search and actually nineteen sequences were found in common with these previous reports, demonstrating the validity of the chosen selection criteria. As their serodiagnostic potential has already been evaluated, these 19 sequences were discarded from our analysis.

Among the database of 87 Map-specific sequences, potentially antigenic proteins were identified using bioinformatic tools. These last steps led to the final selection of three Map-specific candidate antigens, named Ag5, Ag6 and Ag7.

These three genes were cloned and produced as recombinant his-tagged proteins in *E. coli*. Antibody ELISA-based serological test was used to evaluate their ability to discriminate Map infected cattle from negative controls. Ag5 clearly demonstrated insufficient antigenicity since no clear increase in the signal obtained with sera of Map infected animals could be recorded. Results obtained with the two other antigens were clearly more encouraging. Indeed, ELISA-based serological test using Ag6 and Ag7 as coating antigens permitted to reach sensitivities of 72.2% and 83.3%, respectively. The Pourquier test, one of the mostly used tests in Europe, yielded on the same panel of sera, a sensitivity of 72.2% and a Sp of 91.7%. Clearly, ELISA-based serological tests using Ag6 and Ag7 are at least as sensitive as Pourquier test used in standard conditions and should now be evaluated with a larger panel of sera.

Interestingly, the results obtained with Ag6 and Ag7 were better using coating with carbonate than with formaldehyde. In a previous report the positive effect of formaldehyde was clearly demonstrated (Leroy et al., 2007; Speer et al., 2006) and explained by a better fixation of the antigen to the microwell plate through the fixative effect of formaldehyde. However, this explanation is not supported by the observations with Ag6 and Ag7 reported here. It is possible that formaldehyde plays a positive role in antigen detection by stabilisation of some epitopes by covalent cross-link formation. This cross-linking could be detrimental for antibodies directed against certain proteins such as Ag6 and Ag7. In this study, Pourquier test has been used according to the manufactory's protocols without evaluation of formaldehyde treatment. A possible positive effect on sensitivity of the Pourquier test should be evaluated.

It is interesting to note that the sequence of antigen 6 overlaps the complementary strand of MAP0864 and MAP0865 but on the reverse strand, protein sequences thus having no similarities. These two sequences were also selected based on their specificity by Bannantine et al. (2008b). MAP0865 was one of the most immunogenic antigens in the developed protein array analysis. Moreover, in a very recent publication Bannantine et al. (2008a) showed that MAP0865 could be detected as early as 70 days post-infection by sera from experimentally infected animals. Finally, these proteins also gave good results in an ELISA-based test for the diagnosis of ovine paratuberculosis (Bannantine et al., 2008c). It is astonishing that this particular nucleotide sequence leads to two completely different proteins both appearing finally very

immunogenic in independent studies. The expressed sequence of antigen 6 has been verified by mass spectrometry and its peptide mass fingerprint corresponds to the sequence deduced from the nucleotide sequence. Such a precaution was also taken in study involving MAP0865 by sequencing the cloned insert.

The control sera used in this analysis were all from animals from two *M. bovis* infected herds with no known cases of Map infection. Most of the animals were positive in an ESAT6- and CFP10-based interferon gamma test, confirming the within-herd sensitisation to *M. bovis* (Aagaard et al., 2006), further demonstrated by its isolation in half of the cattle. This group of negative control cattle thus represents very stringent discriminating conditions. Facing these negative controls, specificity of the Map Pourquier test was only of 91.6%, *i.e.* four sera among 48 were detected as false-positive. Specificity obtained with Ag6 and Ag7 as coating antigens was 97.9% for both. It means that the strategy chosen here, *i.e. in silico* genomic analysis for selection of Map-specific genes has been efficient. Our results also suggest that when tested with *M. bovis* sensitised animals, the specificity of the Pourquier test decreases significantly in comparison to what is generally observed (>99%; Christopher-Hennings et al., 2003). It is possible that *M. bovis* cross reactivity is not completely abolished by pre-absorption on *M. phlei* extract. The actual specificity of pre-absorbed tests is generally not calculated using *M. bovis* sensitised animals.

Obviously, the use of a 'golden' antigen should be able to discriminate all infected animals regardless of their clinical stage and with a very small false-positive rate. But it is now clear that such an antigen probably does not exist and that the most efficient diagnostic test would be based on a combination of purified antigens. Moreover, Bannantine and coworkers (2008a) have demonstrated, using an experimental infection model, that diagnostic efficiency of an antigen could vary and decrease over the course of the infection. Thus antigens effective in detection of clinical cases could be inefficient for early diagnosis. In this context, antigens presenting sensitivities of more than 70% in an individual test, such as Ag6 and Ag7, must be regarded as good candidates to formulate an antigen combination useful to develop a high efficiency ELISA-based serological test.

Finally, the results obtained in this study clearly indicate that a genomic analysis in search for specificity, combined with a bioinformatic analysis in search of potential immunogenicity is efficient to select candidate antigens. This approach is undoubtedly complementary to the immunoproteomic analysis we have already performed in the same context. Indeed, if the immunoproteomic approach has been shown to be very efficient, not all proteins expressed *in vivo* by Map are sampled in this analysis and thus some very potent antigens might be missed. These antigens could be detected by the genomic *in silico* analysis. On the other hand, immunoproteomic analysis has also shown us that some proteins, even if they are not completely Map specific, could lead to sufficient specificity to be included in a diagnostic test.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2008.09.065.

References

Aagaard, C., Govaerts, M., Meikle, V., Vallecillo, A.J., Gutierrez-Pabello, J.A., Suarez-Guemes, F., McNair, J., Cataldi, A., Espitia, C., Andersen, P., Pollock, J.M., 2006. Optimizing antigen cocktails for detection of *Mycobacterium bovis* in herds with different prevalences of bovine tuberculosis: ESAT6-CFP10 mixture shows optimal sensitivity and specificity. J. Clin. Microbiol. 44, 4326–4335.

Bannantine, J.P., Baechler, E., Zhang, Q., Li, L., Kapur, V., 2002. Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. *avium* reveals potential diagnostic sequences. J. Clin. Microbiol. 40, 1303–1310.

Bannantine, J.P., Barletta, R.G., Stabel, J.R., Paustian, M.L., Kapur, V., 2004a. Application of the genome sequence to address concerns that *Mycobacterium avium* subspecies *paratuberculosis* might be a foodborne pathogen. Foodborne Pathog. Dis. 1, 3–15.

Bannantine, J.P., Bayles, D.O., Waters, W.R., Palmer, M.V., Stabel, J.R., Paustian, M.L., 2008a. Early antibody response against *Mycobacterium avium* subspecies *paratuberculosis* antigens in subclinical cattle. Proteome Sci. 6, 5.

Bannantine, J.P., Hansen, J.K., Paustian, M.L., Amonsin, A., Li, L.L., Stabel, J.R., Kapur, V., 2004b. Expression and immunogenicity of proteins encoded by sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*. J. Clin. Microbiol. 42, 106–114.

Bannantine, J.P., Paustian, M.L., Waters, W.R., Stabel, J.R., Palmer, M.V., Li, L., Kapur, V., 2008b. Profiling bovine antibody responses to *Mycobacterium avium* subsp. *paratuberculosis* infection by using protein arrays. Infect. Immun. 76, 739–749.

Bannantine, J.P., Rosu, V., Zanetti, S., Rocca, S., Ahmed, N., Sechi, L.A., 2008c. Antigenic profiles of recombinant proteins from *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne's disease. Vet. Immunol. Immunopathol. 122, 116–125.

Cetinkaya, B., Egan, K., Harbour, D.A., Morgan, K.L., 1996. An abattoir-based study of the prevalence of subclinical Johne's disease in adult cattle in south west England. Epidemiol. Infect. 116, 373–379.

Cho, D., Shin, S.J., Talaat, A.M., Collins, M.T., 2007. Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins. Protein Expr. Purif. 53, 411–420.

Cho, D., Sung, N., Collins, M.T., 2006. Identification of proteins of potential diagnostic value for bovine paratuberculosis. Proteomics 6, 5785–5794.

Christopher-Hennings, J., Dammen, M.A., Weeks, S.R., Epperson, W.B., Singh, S.N., Steinlicht, G.L., Fang, Y., Skaare, J.L., Larsen, J.L., Payeur, J.B., Nelson, E.A., 2003. Comparison of two DNA extractions and nested PCR, real-time PCR, a new commercial PCR assay, and bacterial culture for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. J. Vet. Diagn. Invest. 15, 87–93.

Collins, M.T., Wells, S.J., Petrini, K.R., Collins, J.E., Schultz, R.D., Whitlock, R.H., 2005. Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. Clin. Diagn. Lab. Immunol. 12, 685–692.

Dupont, C., Thompson, K., Heuer, C., Gicquel, B., Murray, A., 2005. Identification and characterization of an immunogenic 22 kDa exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. J. Med. Microbiol. 54, 1083–1092.

Giese, S.B., Ahrens, P., 2000. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. Vet. Microbiol. 77, 291–297.

Gonda, M.G., Chang, Y.M., Shook, G.E., Collins, M.T., Kirkpatrick, B.W., 2007. Effect of *Mycobacterium paratuberculosis* infection on production,

reproduction, and health traits in US Holsteins. Prev. Vet. Med. 80, 103–119.
Harris, N.B., Barletta, R.G., 2001. *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. Clin. Microbiol. Rev. 14, 489–512.
Huntley, J.F., Stabel, J.R., Bannantine, J.P., 2005. Immunoreactivity of the *Mycobacterium avium* subsp. *paratuberculosis* 19-kDa lipoprotein. BMC Microbiol. 5, 3.
Jakobsen, M.B., Alban, L., Nielsen, S.S., 2000. A cross-sectional study of paratuberculosis in 1155 Danish dairy cows. Prev. Vet. Med. 46, 15–27.
Kennedy, D.J., Benedictus, G., 2001. Control of *Mycobacterium avium* subsp. *paratuberculosis* infection in agricultural species. Rev. Sci. Tech. 20, 151–179.
Leroy, B., Roupie, V., Noel-Georis, I., Rosseels, V., Walravens, K., Govaerts, M., Huygen, K., Wattiez, R., 2007. Antigen discovery: a postgenomic approach to paratuberculosis diagnosis. Proteomics 7, 1164–1176.
Letesson, J.J., Lostrie-Trussart, N., Depelchin, A., 1985. Production d'anticorps monoclonaux spécifiques d'isotypes d'immunoglobulines bovines. Ann. Med. Vet. 129, 131–141.
Li, L., Bannantine, J.P., Zhang, Q., Amonsin, A., May, B.J., Alt, D., Banerji, N., Kanjilal, S., Kapur, V., 2005. The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. Proc. Natl. Acad. Sci. U.S.A. 102, 12344–12349.
McKenna, S.L., Keefe, G.P., Barkema, H.W., Sockett, D.C., 2005. Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. Vet. Microbiol. 110, 105–111.
Olsen, I., Tryland, M., Wiker, H.G., Reitan, L.J., 2001. AhpC, AhpD, and a secreted 14-kilodalton antigen from *Mycobacterium avium* subsp. *paratuberculosis* distinguish between paratuberculosis and bovine tuberculosis in an enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. 8, 797–801.
Ott, S.L., Wells, S.J., Wagner, B.A., 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. 40, 179–192.
Paustian, M.L., Amonsin, A., Kapur, V., Bannantine, J.P., 2004. Characterization of novel coding sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*: implications for diagnosis of Johne's Disease. J. Clin. Microbiol. 42, 2675–2681.
Rosseels, V., Marche, S., Roupie, V., Govaerts, M., Godfroid, J., Walravens, K., Huygen, K., 2006. Members of the 30- to 32-kilodalton mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. *paratuberculosis* are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. Infect. Immun. 74, 202–212.
Roupie, V., Leroy, B., Rosseels, V., Piersoel, V., Noel-Georis, I., Romano, M., Govaerts, M., Letesson, J.J., Wattiez, R., Huygen, K., 2008. Immunogenicity and protective efficacy of DNA vaccines encoding MAP0586c and MAP4308c of *Mycobacterium avium* subsp. *paratuberculosis* secretome. Vaccine 26, 4783–4794.
Sockett, D.C., Conrad, T.A., Thomas, C.B., Collins, M.T., 1992. Evaluation of four serological tests for bovine paratuberculosis. J. Clin. Microbiol. 30, 1134–1139.
Speer, C.A., Scott, M.C., Bannantine, J.P., Waters, W.R., Mori, Y., Whitlock, R.H., Eda, S., 2006. A novel enzyme-linked immunosorbent assay for diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* infections (Johne's Disease) in cattle. Clin. Vaccine Immunol. 13, 535–540.
Streeter, R.N., Hoffsis, G.F., Bech-Nielsen, S., Shulaw, W.P., Rings, D.M., 1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. 56, 1322–1324.
Sweeney, R.W., Whitlock, R.H., McAdams, S., Fyock, T., 2006. Longitudinal study of ELISA seroreactivity to *Mycobacterium avium* subsp. *paratuberculosis* in infected cattle and culture-negative herd mates. J. Vet. Diagn. Invest. 18, 2–6.
Wells, S.J., Wagner, B.A., 2000. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J. Am. Vet. Med. Assoc. 216, 1450–1457.
Willemsen, P.T., Westerveen, J., Dinkla, A., Bakker, D., van Zijderveld, F.G., Thole, J.E., 2006. Secreted antigens of *Mycobacterium avium* subspecies *paratuberculosis* as prominent immune targets. Vet. Microbiol. 114, 337–344.